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Plasma levels of H- and L-ficolin are increased in axial spondyloarthritis: improvement of disease identification

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Summary

Axial spondyloarthritis (axSpA) is a chronic inflammatory disease that primarily affects the axial skeleton. A predominance of innate *versus* adaptive immune responses have been reported in axSpA, indicating a prominent autoinflammatory component of the disease. Little is known about the lectin pathway proteins (LPPs) of the complement system in relation to axSpA. We have investigated LPPs in patients with axSpA and control individuals. Plasma samples were obtained from a cross-sectional cohort of 120 patients with a clinical diagnosis of axSpA and from 144 age- and gender-matched controls. The plasma concentrations of 11 LPPs were measured, using sandwich-type time-resolved immunofluorometric assays in patients and controls, and related to clinical diagnosis and disease activity. Three LPPs [H-ficolin (ficolin-3), L-ficolin (ficolin-2) and collectin liver 1 (CL-L1)] were significantly higher in axSpA patients than in controls ($P < 0{\cdot}0001$) and one LPP, collectin kidney 1 (CL-K1), was significantly lower ($P < 0{\cdot}0001$). Further, combining H- or L-ficolin concentrations above the 75th percentile of the respective H- or L-ficolin concentration measured in controls with human leucocyte antigen (HLA)-B27 positivity yielded axSpA diagnostic specificities of 99/99% and positive likelihood ratios of 68/62, respectively. H-ficolin and L-ficolin plasma concentrations were found to be elevated in axSpA patients regardless of time since diagnosis. H-ficolin and L-ficolin may represent diagnostic biomarkers for patients with axSpA and should be further evaluated. Our results showed no association between disease activity and the measured LPP concentrations. This result might be due to the cross-sectional design, and should be further investigated.

Keywords: axial spondyloarthritis, complement pathway, ficolins, innate immunity, lectin proteins

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Introduction

Axial spondyloarthritis (axSpA) is a chronic inflammatory disease that primarily affects the axial skeleton. It is a type of spondyloarthritis which also includes psoriatic arthritis, arthritis associated with inflammatory bowel disease and reactive arthritis. The term 'axSpA' covers both patients who have developed structural damage in the sacroiliac joints or the spine visible on radiographs (radiographic axSpA or ankylosing spondylitis (AS) and patients without such structural damage (non-radiographic axSpA) [1].

The diagnosis of axial spondyloarthritis (axSpA) relies on the presence or absence of a combination of symptoms, clinical and biochemical findings and sacroiliitis on imaging [1]. However, the diagnosis of axSpA is complicated, particularly in early disease stages or when conditions mimicking the findings in axSpA co-exist [2]. Due to the lack of unambiguous clinical symptoms and specific biomarkers, the diagnosis often relies on the presence of human leucocyte antigen-B27 (HLA-B27) and/or magnetic resonance imaging (MRI) findings [3]. However, the identification of a 'positive MRI' in axSpA remains controversial, as both sensitivity and specificity show limitations, and

the interpretation of MRI lesions in daily practice is critically dependent on the clinical context [4].

A predominance of innate *versus* adaptive immune responses have been reported in axSpA, indicating a prominent autoinflammatory component of the disease [5]. The complement system is part of the innate immune system and is activated through three pathways: the classical, the alternate and the lectin pathway. All these pathways lead to the activation of C3 and thereby initiation of the common pathway ending in membrane attack complex formation [6]. The complement system participates in regulation of the adaptive immune system and in the removal of immune complexes and cell debris. The pattern recognition molecules of the lectin pathway (LP) of the complement system recognize abnormal surface patterns, initiate killing of pathogens and eliminate damaged cells through opsonization and activation of the complement system [7]. Increasing evidence supports that failure to remove apoptotic cells is associated with autoimmune disease [8]. The present report concerns the evaluation of the 11 proteins [mannan-binding lectin (MBL), collectin liver-1 (CL-L1), collectin kidney-1 (CL-K1), H-ficolin (alternative name; ficolin-3), L-ficolin (ficolin-2), M-ficolin (ficolin-1), MBL-associated serine proteases (MASP)-1, -2 and -3 and MBL-associated proteins of 19 and 44 kDa (MAp19 and MAp44, respectively)], constituting the lectin pathway of the complement system in relation to axSpA.

Little is known about the complement system in relation to axSpA. In one study, inhibition of complement component 3 (C3) in a mouse model of AS seemed to delay the progression of the disease [9]. Of the lectin pathway proteins (LPPs), only MBL has been investigated in relation to axSpA. Haplotypes of MBL2-gene polymorphism were found to be associated with AS, and MBL deficiency seemed more frequent in Brazilian axSpA patients [10].

Studies in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) have shown that the innate immune system, including the LP, plays a role in the pathogenesis [11–13]. These results suggest that activation of the LP could be a common risk factor in chronic inflammatory diseases.

The objective of our study was to measure plasma concentrations of LPPs in patients with axSpA and control individuals and to analyse for their involvement in axSpA pathogenesis and their potential for early identification of axSpA.

Materials and methods

Patient population

A cross-sectional cohort of 120 patients with a clinical diagnosis of axSpA was diagnosed by a rheumatologist and validated by an experienced rheumatologist (A.G.L.) as a gold-standard diagnosis, incorporating all clinical, laboratory and imaging data for the evaluation. All the patients fulfilled the ASAS 2009 criteria [1]. The patients were recruited at the out-patient clinic of the Department of Rheumatology, Aarhus University Hospital (AUH) 2015–17. Exclusion criteria were age < 18 years, past or present malignancy, psoriasis, diagnosed inflammatory bowel disease or other chronic inflammatory diseases. Patient characteristics and disease measures were registered on the day of inclusion. Age- and gender-matched control individuals (n = 144) were recruited from the blood bank at AUH. All participants gave written informed consent.

Blood sampling and biochemical analyses

Peripheral venous blood samples were obtained at the day of inclusion for both patients and controls in ethylenediamine tetraacetic acid (EDTA) collecting tubes. Blood samples were centrifuged at 2000 *g* for 10 min at room temperature, and plasma was collected and stored at –80°C until analysis.

The concentration of all 11 proteins of the lectin pathway (MBL, CL-L1, CL-K1, M-ficolin, H-ficolin, L-ficolin, MASP-1, -2 and -3, MAp19 and MAp44), as well as complement fragment factor C3 and the activation fragment C3dg, were analysed in EDTA plasma using time-resolved immunofluorometric assays at the Department of Biomedicine, Aarhus University, as previously described [14,15]. Briefly, plasma was thawed, diluted in assay buffers and added to microtitre wells coated with relevant capture antibodies or mannan (for the MBL assay) or acetylated bovine serum albumin (for the H-ficolin assay). In-house biotinylated antibodies followed by europium labelled streptavidin were used for detection of proteins caught in the wells. The signals obtained in the wells were compared to a standard curve of known protein content, and each microtitre plate contained three quality controls. The intra- and interassay coefficients of variation were below 15% for all assays. The person performing the analysis was blinded to clinical information at the time of analysis.

L-ficolin was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) assay (Hycult Biotech, Uden, the Netherlands; #HK336-02), as instructed by the manufacturer.

Serological typing of HLA-B27 was performed by the use of a complement-dependent microlymphocytotoxicity test in accordance with the European Federation of Immunogenetics' Standards for Histocompatibility and Immunogenetics Testing [16] and was conducted as previously described [17]. This was the standard method at the time of inclusion at the Department of Clinical Immunology, AUH. The serological method has been shown to yield results similar to the more recent polymerase

chain reaction (PCR) method, including similar sensitivity and specificity [18].

Statistical analysis

The Mann–Whitney *U*-test was used to compare plasma levels of the proteins in patients and controls. Correlation analyses were performed calculating Spearman's rank correlation. *P*-values < 0·05 were considered statistically significant. The Bonferroni correction was used to adjust for multiple testing.

Ethical aspects

The study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Regional Committee on Health Research Ethics (case no. 1-10-72-307-15) and the Danish Data Protection Agency (jr.nr. 1-16-02-18-16).

Results

Patient characteristics

The patients in the study cohort had a broad age range (median = 36·5; range = 18–72), 85% were HLA-B27-positive, 61% were male, 69% of the patients fulfilled the New York criteria for ankylosing spondylitis [19], 13% had peripheral arthritis at inclusion (53% had ever had peripheral arthritis) and 21% had associated uveitis. The median time since first diagnosis was 2·6 years (range = 0–40·8 years) and the median time since first symptom was 10·7 years (range = 0·3–46 years). The disease activity was relatively high [mean ± standard deviation (s.d.); Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) = 38·6 ± 23·3; Ankylosing Spondylitis Disease Activity Score (ASDAS) = 2·5 ± 1·14, respectively], with 65% of the patients using non-steroidal anti-inflammatory drugs (NSAID) and 45% being on tumour necrosis factor (TNF)-α inhibitor at inclusion (Adalimumab 11%; Certolizumab 4%; Etanercept 41%; Golimumab 9% and Infliximab 35%, respectively) (Table 1). Controls had a median age of 41 years (range = 19–67 years).

LPP concentrations in plasma of axSpA patients and controls

Plasma concentrations of LPPs differed significantly between axSpA patients and controls for all proteins except M-ficolin, MAp19 and MAp44 (Table 2). However, when adjusting for multiple testing (Bonferroni *P*-value 0·004) only H-ficolin, L-ficolin and CL-L1 levels remained significantly higher in axSpA patients than in controls ($P < 0·001$) and CL-K1 levels were significantly lower ($P < 0·001$) (Fig. 1).

Table 1. Demographics

Patient characteristics	Spondyloarthritis, *n* = 120
Median age, years (range)	36·5 (18–72)
Proportion males, *n* (%)	73 (61%)
Proportion with ankylosing spondylitis, *n* (%)	83 (69%)
Proportion HLA-B27-positive, *n* (%)	102 (85%)
Proportion with uveitis, *n* (%)	25 (21)
Proportion with peripheral arthritis at inclusion, *n* (%)	16 (13%)
Median time since first diagnosis, years (range)	2·6 (0–40·8)
Median time since first symptom, years (range)	10·7 (0·3–46)
ASDAS*, mean (s.d.)	2·5 (1·14)
BASDAI†, mean (s.d.)	38·6 (23·3)
BASFI‡, mean (s.d.)	30·2 (25·0)
BASMI§, mean (s.d.)	15·1 (19·7)
VAS** pain (0–100), mean (s.d.)	40·4 (28·2)
VAS fatigue (0–100), mean (s.d.)	48·7 (29·2)
VAS global patient (0–100), mean (s.d.)	46·1 (29·8)
VAS global physician (0–100), mean (s.d.)	19·0 (17·2)
Proportion using NSAID†† at inclusion, *n* (%)	78 (65)
Proportion using DMARD‡‡ at inclusion, *n* (%)	24 (20)
Proportion using anti-TNF-α at inclusion, *n* (%)	54 (45)
Type of anti-TNF-α used at inclusion, Ada/Cer/Eta/Gol/Inf§§, respectively, *n* (%)	6(11)/2(4)/22(41)/5(9)/19(35)
BMI***, mean (s.d.)	25·2 (5·0)
Current smokers, *n* (%)	27 (22·5)
Ever smokers, *n* (%)	56 (46·7)

*ASDAS = Ankylosing Spondylitis Disease Activity Score; †BASDAI = Bath Ankylosing Spondylitis Activity Index; ‡BASFI = Bath Ankylosing Spondylitis Functional Index; §BASMI = Bath Ankylosing Spondylitis Metrology Index; **VAS = visual analogue scale; ††NSAID = non-steroidal anti-inflammatory drugs; ‡‡DMARD = disease-modifying anti-rheumatic drugs; §§Ada/Cer/Eta/Gol/Inf = Adalimumab, Certolizumab, Etanercept, Golimumab, infliximab; ***BMI = body mass index; TNF = tumour necrosis factor; s.d. = standard deviation.

Table 2. Plasma lectin pathway protein concentrations in patients with axial spondyloarthritis (axSpA) and in controls

Lectin pathway protein	AxSpA* $n = 120$	Controls $n = 144$	P
MBL† μg/ml, median (range)	2·2 (0–9·2)	1·7 (0–6·3)	0·04
M-ficolin μg/ml, median (range)	4·5 (2·5–10·1)	4·5 (1·7–8·2)	0·17
L-ficolin μg/ml, median (range)	4·2 (0·77–13·5)	2·7 (1·1–6·0)	< 0·0001
H-ficolin μg/ml, median (range)	30·7 (8·3–64·0)	19·8 (8·3–40·9)	< 0·0001
CL-L1‡ μg/ml, median (range)	0·62 (0·4–1·1)	0·59 (0·3–0·8)	0·0002
CL-K1§ μg/ml, median (range)	0·41 (0·3–0·7)	0·46 (0·2–0·6)	< 0·0001
MASP**-1 μg/ml, median (range)	9·7 (4·2–12.2)	10·8 (4·8–37·7)	0·005
MASP-3 μg/ml, median (range)	9·1 (3·1–20·0)	8·4 (4·2–18·0)	0·02
MAp44†† μg/ml, median (range)	2·7 (1·3–6·4)	2·7 (1·3–4·5)	0·50
MASP-2 μg/ml, median (range)	0·53 (0–1·7)	0·49 (0·1–1·3)	0·01
MAp19‡‡ μg/ml, median (range)	0·48 (0·2–0·8)	0·47 (0·3–0·6)	0·18

*axSpA = axial spondyloarthritis; †MBL = mannan-binding lectin; ‡CL-L1 = collectin liver-1; §CL-K1 = collectin kidney-1; **MASP = mannan-binding lectin associated serine protease; ††MAp44 = MBL-associated protein of 44 kDa; ‡‡MAp19 = MBL-associated protein of 19 kDa.

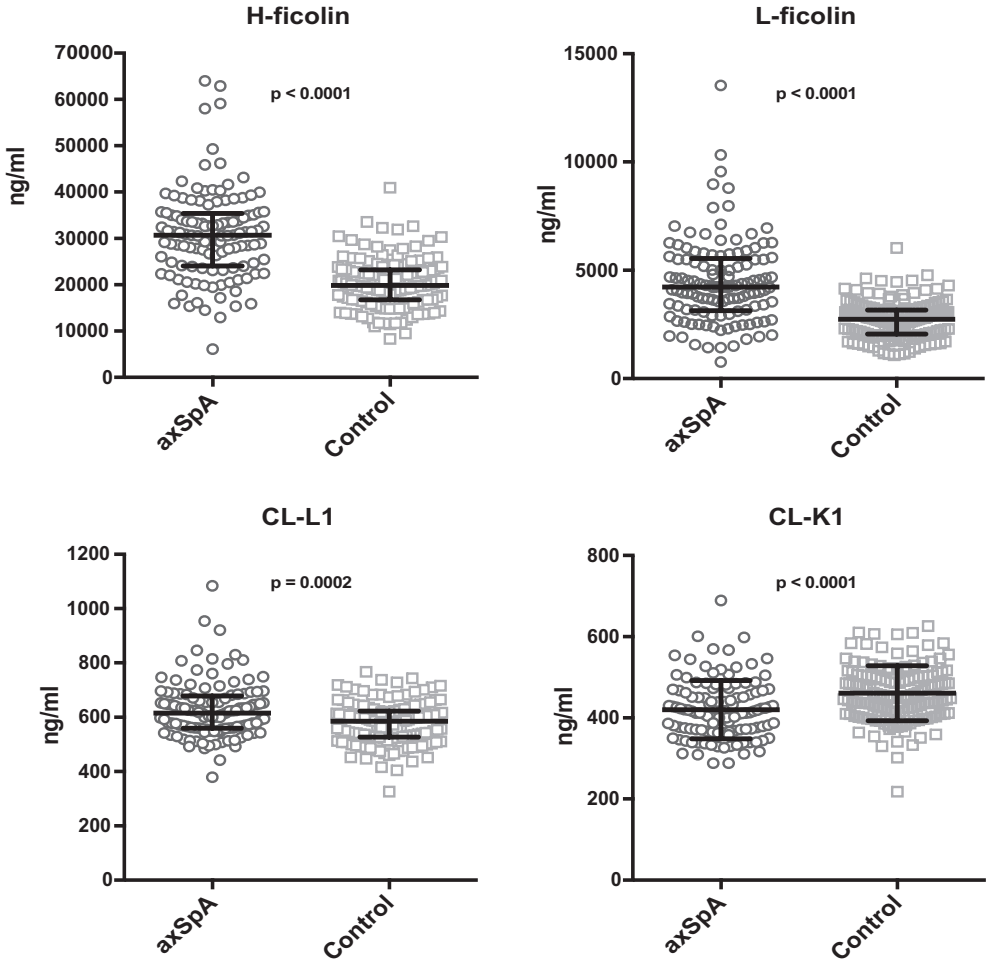

Fig. 1. Plasma concentrations of lectin pathway proteins in patients with axial spondyloarthrtis (axSpA; black circles) and in control individuals (grey squares). Bars indicate median and interquartile range. CL-L1 = collectin liver-1; CL-K1 = collectin kidney-1.

LPP and disease activity

No significant association was observed between disease activity measured by ASDAS or BASDAI and H-ficolin, L-ficolin, CL-L1 and CL-K1, respectively (Fig. 2). Moreover, no significant associations were found between changed protein concentration and manifestations such as uveitis,

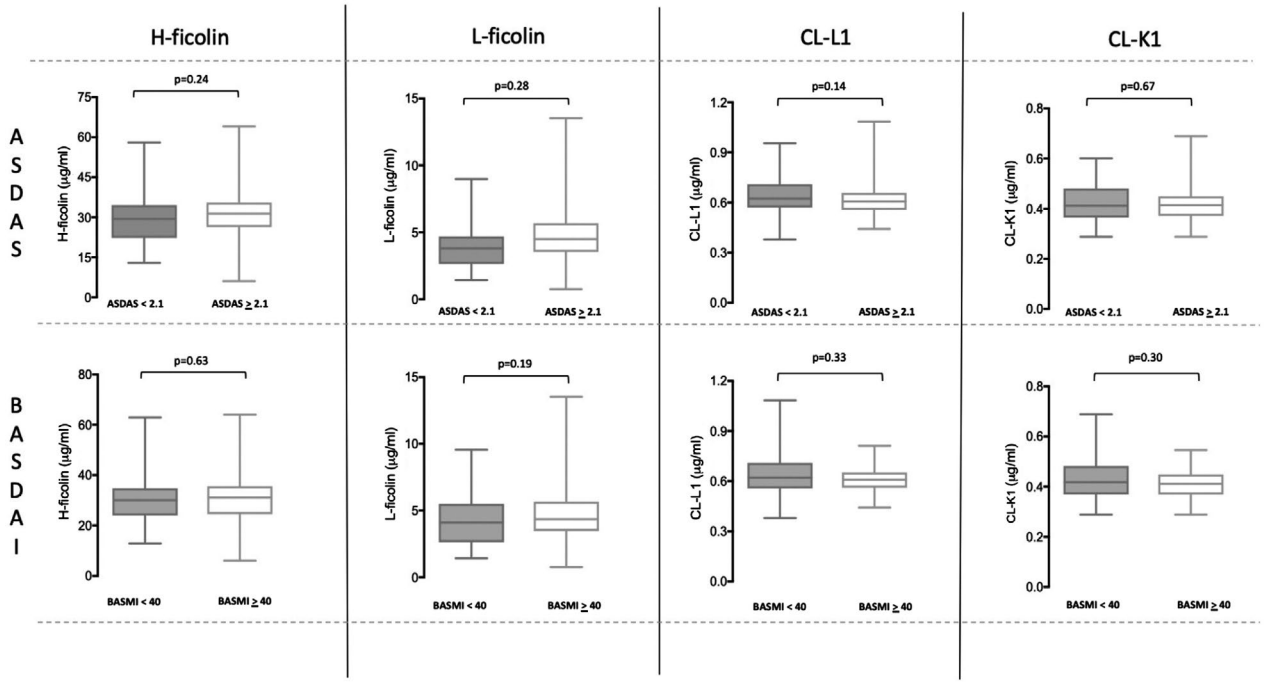

Fig. 2. Association between lectin pathway proteins and disease activity scores for proteins where a significant difference was observed between patients with axial spondyloarthritis and control individuals after adjusting for multiple testing. Bars indicate median and interquartile range. ASDAS = Ankylosing Spondylitis Disease Activity Score; BASDAI = Bath Ankylosing Spondylitis Disease Activity Index.

radiographic changes, time since diagnosis, CRP, C3, C3dg and different treatment regimens (data not shown).

LPPs as biomarkers for axSpA diagnosis

H-ficolin and L-ficolin showed the largest difference between axSpA patients and controls. For these proteins we stratified the plasma levels based on the 75th percentiles measured in the controls and HLA-B27 into positive or negative, yielding the distribution of axSpA patients and controls shown in Fig. 3.

Sensitivity, specificity, likelihood ratios and predictive values were calculated for each protein and for the combination of proteins (Table 3). H- or L-ficolin above the 75th percentile (based on the value from controls) combined with HLA-B27 positivity resulted in specificities of 99/99% and positive likelihood ratios (LR+s) of 68/62, respectively, for the axSpA diagnosis.

Complement activation

No sign of complement activation, as estimated from complement activation fragment C3dg concentration, was found comparing axSpA patients with controls (Fig. 4).

Discussion

This study is the first, to our knowledge, to quantify all LPPs in axSpA patients. LPPs, particularly H- and L-ficolin, were found elevated in axSpA. No association was observed with disease activity or complement activation. However, combining HLA-B27 positivity with an elevated level of H- or L-ficolin increased the diagnostic specificity for axSpA.

HLA-B27 is a key factor in the axSpA diagnosis [3]. Although highly sensitive for the diagnosis it is unspecific, as approximately 10% of healthy individuals are positive. We observed that the diagnostic specificity of HLA-B27 could be improved by combining it with either elevated H- or L-ficolin concentrations yielding LR+s of 68/62, respectively. When comparing these likelihood ratios with previously published LR+s in ankylosing spondylitis [20], where a LR+ of 9·0 was reported for HLA-B27 and the highest LR+ of 20 was reported for sacroiliitis grade 3, the present results appear promising as new biomarkers for the diagnosis of axSpA.

Even when used as an individual biomarker, an elevated level of H-ficolin or L-ficolin might be a valuable addition in the diagnosis of axSpA where there are no diagnostic criteria. Interpretation of MRI can be difficult, and MRI has shown limited sensitivity and specificity in axSpA diagnosis [4]. In particular, early diagnosis of axSpA has been difficult due to the late occurrence of definite imaging findings [21]. The finding of an elevated level of H-ficolin or L-ficolin in axSpA patients, independent of time since diagnosis or radiographic findings, indicates a

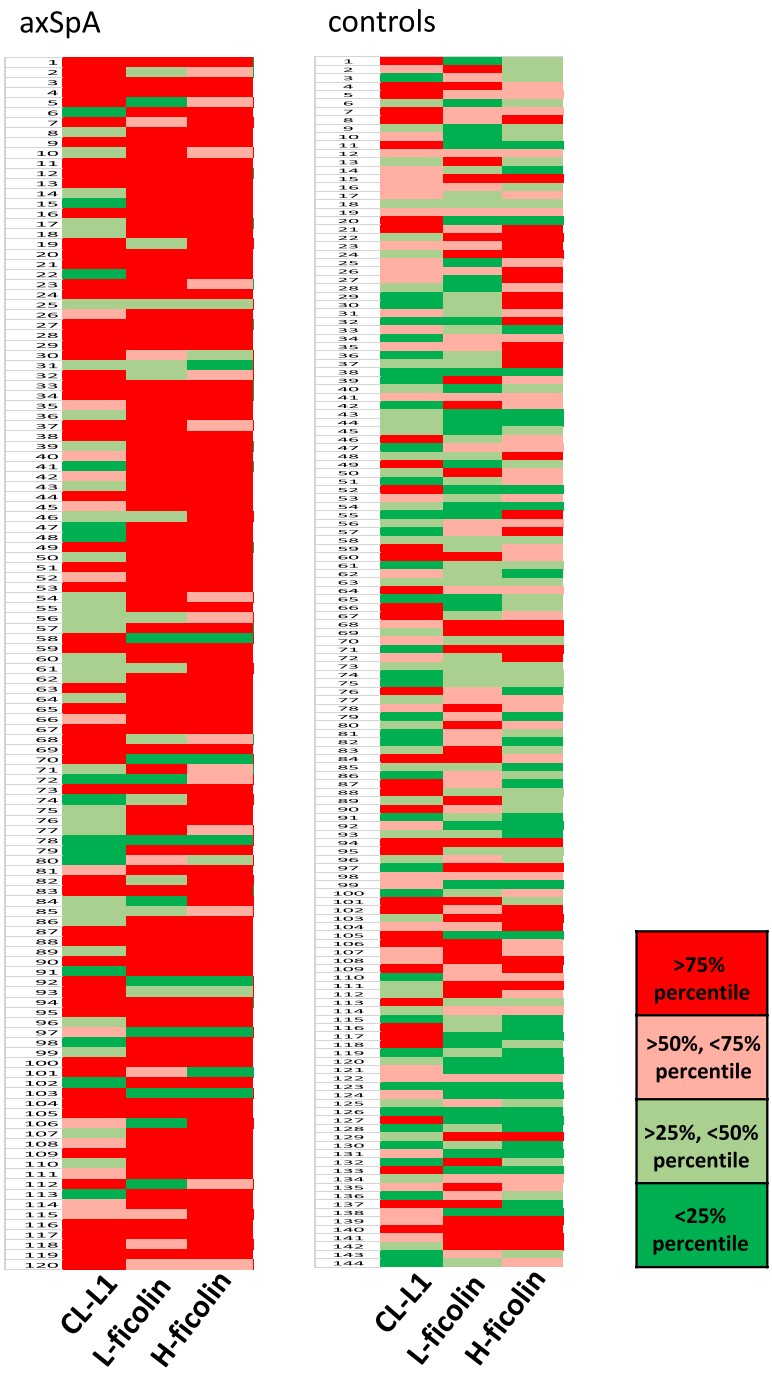

Fig. 3. Lectin pathway proteins in axial spondyloarthritis (axSpA). Heat map of the plasma concentrations of L-ficolin (ficolin 2) and H-ficolin (ficolin 3) and human leucocyte antigen (HLA)-B27 results from 120 individual axSpA patients and 144 controls. Green colour represents the lowest 25th percentile; light green represents plasma concentrations from the 25th percentile to the median concentration; pink represents plasma concentrations from median to the 75th percentile; and red represents the highest concentration above the 75th percentile. For HLA-B27, red represents the presence of HLA-B27 and green the absence. The percentiles for the axSpA patients are based on the concentrations measured in the 144 control individuals.

potential use of these proteins as biomarkers in the deductive process of an early diagnosis of axSpA.

The LPP patterns reported in SLE and RA [11,13,15,22] differ from that found in our axSpA cohort. This possibly reflects that different parts of the innate immune system are involved in each of these diseases and might be due to that both SLE and RA are humoral-associated autoimmune diseases, where autoantibodies precede the clinical

Table 3. The diagnostic potential of H-ficolin, L-ficolin, HLA-B27 and a combination of these in axial spondyloarthritis

HLA-B27 and lectin pathway biomarkers in axial spondyloarthritis

	One biomarker present			Two biomarkers present			Three biomarkers present
	H-ficolin	L-ficolin	HLA-B27	H-ficolin + L-ficolin	H-ficolin + HLA-B27	L-ficolin + HLA-B27	H-ficolin + L-ficolin + HLA-B27
Sensitivity (%)	78	74	85	69	68	62	60
Specificity (%)	76	76	94	82	99	99	99
LR+*	3·3	3·1	14·2	3·8	68	62	60
LR–†	0·3	0·3	0·16	0·38	0·32	0·38	0·4
PPV‡ (%)	73	72	92	99	99	99	99
NPV§ (%)	80	78	88	75	79	76	76

Diagnostic biomarker potential of H-ficolin (ficolin 3) and L-ficolin (ficolin 2) above the 75th percentile of the controls and a positive human leucocyte antigen (HLA)-B27, either alone or in combination. Sensitivity, specificity; *positive likelihood ratio (LR+); †negative likelihood ratio (LR–); ‡positive predictive value (PPV) and §negative predictive value (NPV) was estimated.

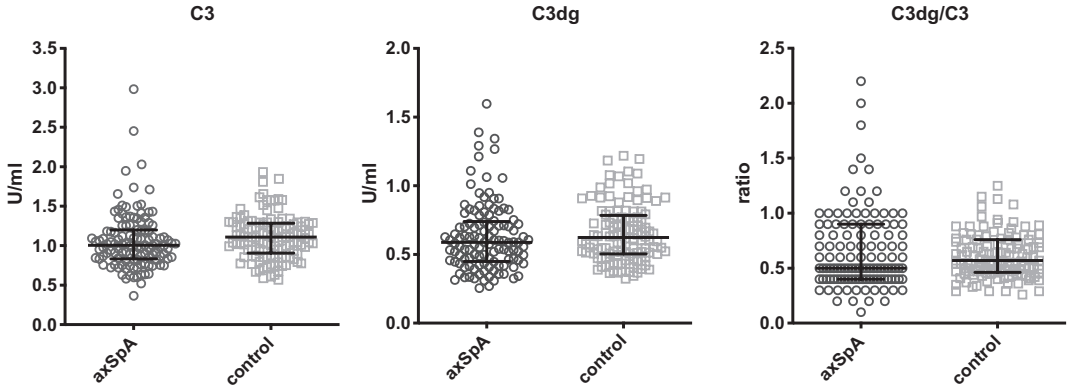

Fig. 4. Plasma concentrations of complement proteins C3 and C3dg in patients with axial spondyloarthritis (axSpA; black circles) and in controls (grey squares). Bars indicate median and interquartile range.

presentation of the disease. In contrast to this, in axSpA there is increasing evidence that mechanical stress triggers recurrent tissue microdamage [23,24]. This microdamage, in combination with innate immune dysregulation, contributed to by gut barrier dysfunction, may result in entheseal immune activation [25], which is a cornerstone of axSpA pathogenesis. These differences in immune mechanisms may influence the synthesis and consumption of H- and L-ficolin and CL-L1 in relation to axSpa pathogenesis and may explain our observation of no complement activation in axSpA patients, whereas immense activation is observed in SLE [15]. It is possible that the changes observed in the pattern recognition molecules in axSpA patients represents a function of the pattern recognition molecules in tissue homeostasis which is not associated with complement activation. However, further studies are needed to elucidate this hypothesis.

This study is the first to investigate the role of ficolins and other pattern recognition molecules in axSpA and demonstrates a potential interesting role in the pathogenesis of the disease. Whether the changes in H- and L-ficolin concentration are the result of the disease or the cause leading to the disease cannot be answered by the present study design.

A strength of the present study is the well-characterized group of patients with axSpA. However, for future studies a longitudinal study of a cohort of patients with a suspected diagnosis of axSpA followed systematically over time to confirm the diagnosis is needed to validate the diagnostic value of increased plasma level of H- or L-ficolin combined with HLA-B27 positivity.

In summary, this is the first report of elevated plasma levels of LPPs in axSpA. An elevated plasma H- or L-ficolin improves the diagnostic specificity of HLA-B27

positivity. Because H- and L-ficolin exhibited high plasma concentrations regardless of time since diagnosis, H- or L-ficolin may be new promising biomarkers for the diagnosis of axSpA. A biomarker for axSpA diagnosis is urgently needed in cases of back pain where the interpretation of MRI is difficult [4]. This might be the case both in early diagnosis, where no significant MRI lesions can be identified [4], and in cases where the interpretation of the clinical information related to diagnosis is complicated [2].

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Disclosures

All authors have no conflicts of interest to declare.

Author contributions

A. G. L., A. T. and C. E. M. contributed to design, acquiring and analysis of the data, statistical calculations as well as writing the manuscript. S. T. and K. S.-P. contributed to design and analysis of data. A. T., C. E. M., T.-L. K. and A. H. conducted laboratory experiments. A. G. L., A. T. and K. S.-P. obtained the grant for the study. All authors revised and approved the manuscript for submission.

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